0022-202X/7805-0414$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 78:414–424, 1982

Vol. 78, No. 5
Printed in U.S.A.

Ultrastructural, Histochemical, and Biochemical Studies of the Melanin Metabolism in Eye and Skin of Pallid Mice

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The hair follicles and the eyes of pallid mice (C57/6J-Pa/Pa) and those of black mice (C57/6J−+/Pa) were examined ultrastructurally, histochemically, and biochemically to determine the cause of pigment dilution.

The pigment cells in the hair follicles and the eyes of pallid mice have less mature melanosomes than those of black mice. In the hair follicles the pallid melanosomes were transferred into keratinocytes and became aggregated. In the eyes they were already aggregated within the pigment cells and were digested in acid phosphatase-positive lysosomes. The activity of acid phosphatase, a marker of lysosomal enzymes, was significantly higher in pallid hair follicles and eyes than in black hair follicles and eyes.

Dopa reactions at light and electron microscopical levels indicated that the pigment cells in each tissue produced a large amount of Dopa oxidase when compared with those in each black counterpart. However, the rate of hydroxylation of L-tyrosine−3,5−^{3}H was significantly lower in the pallid eyes than in black eyes, while this rate was significantly higher in pallid hair follicles than in black hair follicles. Immediate digestion of melanosomes within the pigment cells, i.e., autophagocytosis, seemed to explain the low activity in the pallid eyes.

The diluted coat and eye colors of pallid mice are, therefore, not related to low Dopa oxidase activity but to immaturity of melanosomes and high activities of lysosomal enzymes; these enzymes seem to digest many of these immature melanosomes and contribute to the diluted coat and eye colors of pallid mice.

Although skin, hair, and eye colors are determined by genetic factors, the mechanisms of phenotype expression have not been clarified. There are several possible mechanisms regulating those colors: (I) population and distribution of melanocytes, (II) number and structure of melanosomes, (III) activities of melanin synthesizing enzymes, (IV) action of hormonal factors or of melanotrophic nerves, and (V) interaction between melanocytes and keratinocytes, i.e., melanosome transfer.

This study added another possible mechanism by presenting evidence that the active digestion of melanosomes by lysosomes is related to the pigment dilution of coat and eye colors in a mouse. For experimental models, pallid mice (C57/6J-Pa/Pa), which have a peculiar diluted coat color and dark red eyes, were examined ultrastructurally, histochemically, and biochemically.

Manuscript received December 31, 1980; accepted for publication October 29, 1981

This work was supported by in part by a research grant by the Veterans Administration Center, Dayton, OH and by a research grant EY-01959 from the NIH for Wright State University, Dayton, OH.

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Abbreviations:
PNPP: para-nitrophenylphosphate
RPE: retinal pigment epithelium

MATERIALS AND METHODS

Materials

Mice originally derived from C57/6J-Pa/Pa inbred strain maintained by the Jackson Laboratory (Bar Harbor, ME) were obtained from Dr. L. C. Erway at the University of Cincinnati. The pallid gene was maintained by backcrossing Pa/Pa and +/Pa genotypes in the animal facility at Wright State University. In this study +/Pa (black) littermate mice were used as control. Since light may influence the level of lysosomal enzymes in eye tissues [1] all mice were maintained on a 12 hr light-12 hr dark cycle.

Skin was taken from the backs of 7-day-old mice; hair was in anagen cycle. Skin of adult mice (120-days-old) was also taken without using any drugs. Anagen was induced in the skin of adult mice (120-days-old) by plucking hairs 2 weeks before biopsy. The skin was taken from the plucked area. All skin samples for electron microscopical studies were from adult mice. The eyes of 7-day-old* mice were also taken after termination in a chloroform saturated chamber.

For the biochemical assays of tyrosinase and acid phosphatase activities, the anagen hair follicles and the eyes of 7- and 8-day-old mice were used.

Dissection, Fixation and Sectioning

Some of the materials were immediately fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 4 hr. The former was used for ultrastructural demonstration of dopa or acid phosphatase reactions and the latter for fine ultrastructural observation. For the light microscopic studies materials were frozen without fixation and sectioned to about 3 μm thickness in a cryostat at 30°C. Corresponding materials from a pallid mouse and a black mouse were sectioned parallel to each other and mounted on a glass slide side by side to assure the same thickness for quantitative analysis of histochemical reactions. Sections were dried at room temperature, kept in a freezer overnight and stained for dopa or acid phosphatase reaction.

Light Microscopic Dopa Reaction Technique

The sections were rinsed in 0.1 M phosphate buffer (pH 6.8 or 7.4)† and incubated in 0.2% D, L-dopa (D, L-dihydroxyphenylalanine) in the same buffer solution for 3.5 hr at 37°C. This solution was changed every hour during the incubation period to avoid auto-oxidation of dopa and, therefore, nonspecific staining of specimen. The control sections were incubated in dopa-free solution at the same time. After the incubation, sections were rinsed in the same buffer, dehydrated in alcohol and xylene and embedded in synthetic media.

Light Microscopic Acid Phosphatase Reaction

α-Naphthyl phosphate-hexazonium pararosanilin technique [3] was used. Stock solutions were prepared as follows: (a) pararosanilin solution (1 g pararosanilin hydrochloride, 20 ml distilled water, 5 ml concentrated HCl), (b) 4% sodium nitrite in distilled water, and (c) substrate solution (100 mg sodium α-naphthyl phosphate, 25 ml Michaelis Veronal buffer stock solution). The mixture of 0.8 ml of (a), 0.8 ml of (b), 5 ml of (c) and some distilled water was prepared, adjusted to pH 6.0 with 1 N NaOH and diluted with distilled water to the final volume of 20 ml.

The sections were inbuated in this mixture for 30 min at room temperature, stained with 1% methyl green in Veronal buffer (pH 4.0), rinsed in distilled water, dehydrated in alcohol and xylene and em-

* Since mouse eyes were still developing at this age, melanosomes of early stages were studied in these specimens.

† Since King, Olds, and Witkop [2] reported that the tyrosinase of hair bulbs had maximum activities at around pH 7.8, the dopa reactions were performed at both pH 6.8 and 7.4.

bedded in synthetic media. The control sections were incubated in the same mixture to which 0.05 M sodium fluoride (a specific inhibitor of acid phosphatase (4)) had been added or in the mixture in which the substrate (α-naphthyl phosphate) had been omitted.

Positive reaction was indicated by red to reddish-brown precipitate which represents the azo-dye produced by coupling of hexazonium pararosanilin and α-naphthol.

Electron Microscopic Dopa Reaction Technique

The tissues fixed with 3% glutaraldehyde were rinsed and incubated as for light microscopy: 2.5 hr for skin and 4.5 hr for eye tissues.

Electron Microscopic Acid Phosphatase Reaction Technique

Two modified methods of Gomori's technique were employed. In the first method [5] the incubation medium contained 0.5 M acetate buffer (pH 5.0) with 7.5% sucrose, 0.0375% lead acetate and 0.075% sodium β-glycerophosphate as substrate. In the second method [3] the incubation medium contained 0.067 M tris-maleate buffer (pH 5.0), 0.067% lead nitrate and 0.21% sodium β-glycerophosphate as substrate. This medium was incubated at 40°C until turbidity occurred and filtered before use.

In either technique, the tissue pieces fixed with 3% glutaraldehyde were rinsed in the individual buffer overnight and incubated in medium for 30 min at 37°C. The control pieces were incubated in the medium in which the substrate had been omitted, or incubated in the substrate medium to which 0.02 M sodium fluoride had been added.

Procedures for Electron Microscopic Observation

All materials for electron microscopal observation were postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 hr, block-stained in 1% uranyl acetate in 50% ethanol solution for 20 min, dehydrated in graded concentrations of alcohol and propylene oxide, and embedded in Araldite. The ultrathin sections were cut with diamond knives in Ultracut Microtomes (American Optical) and observed in Phillips-400 electron microscope. All sections were double-stained with 15% uranyl acetate in 100% methanol and Reynolds' lead citrate [6].

Preparation of Hair Follicle Samples for Biochemical Assays

Entire back skins of 7-day-old and 8-day-old mice were placed dermis-side up under a stereomicroscope and the muscle layers under adipose tissue were removed. The hair bulbs with hair shafts were plucked from the adipose tissue side (hair root side) with sharp tweezers.‡ The tissue surrounding hair bulbs was removed as much as possible on a filter paper under a stereomicroscope. Hair follicles with hairs were homogenized in 0.1 M phosphate buffer with 0.5% Triton X-100 (pH 6.8) at 0°C for tyrosinase assay or in 0.1 M citric acid buffer with 0.1% Triton X-100 (pH 4.8) at 0°C for acid phosphatase assay. Keratinized portion of hair was allowed to precipitate from homogenates kept in ice for 5 min while the hair bulbs and nonkeratinized proximal hairs were easily separated and used for assays. This separation was confirmed by light microscopic observations.

Preparation of Eye Samples for Biochemical Assays

Eyes were enucleated and lenses were removed from littermate pairs of pallid (Pa/Pa) and black (+/Pa) mice. The entire tissue of enucleated eyes were homogenized in the same ways as hair samples. Following homogenization, the sclera which remained intact were removed and the remaining materials were rehomogenized. Protein was determined according to the method of Lowry et al [7].

Biochemical Assay for Tyrosinase Activity

The tyrosine hydroxylase activity of the aliquots was determined by the method of Pomerantz [8], modified by King, Olds, and Witkop [2] and Dryja et al [9]. An aliquot of 20 μl was added to 40 μl of 0.1 M phosphate buffer (pH 6.8) with 1 μCi L-tyrosine-3,5-^{3}H dried before use and 2.5 MM L-Dopa in a tube. Tubes were incubated in a water bath at 37°C for 30 min with shaking every 5 min and then in ice to stop the reaction. 20 μl of a reacted solution was washed with 1.6 ml of 0.1 M citric acid through a 3.0 cm Dowex 50 W (dry mesh 200–400, 12% cross-linked) column and the total wash was counted in 15 ml scintillation fluid (Aquasol, New England Nuclear). The average counts of blank reactions containing no added hair follicle or eye homogenate were subtracted from each assay value of samples. Results were expressed as counts per min (cpm) per mg protein ± SD. As another control, 1-phenyl-2-thiourea (6.7×10^{-8} M) (a specific inhibitor for tyrosinase [9]) was added to a sample before incubation. All of the samples and controls were determined in duplicate.

Biochemical Assay for Acid Phosphatase Activity

The acid phosphatase activity of the aliquots was measured by the method of Cotman and Matthews [10], which employs hydrolysis rate of para-nitrophenylphosphate (PNPP) at 37°C for 30 min in 1 ml reaction volumes. The optical densities of identical sample boiled for 5 min (enzyme blanks) were subtracted from sample and total moles of PNPP hydrolyzed were determined from standard para-nitrophenol standards (OD 400 nm). Results were expressed as nanomoles of PNPP hydrolyzed per min per mg protein ± SD.

RESULTS

Light Microscopical Studies

Comparison of natural condition of melanin pigments in black and pallid mice: The untreated and unstained frozen skin (Fig 1-*A*) and eye sections (Fig 1-*C*) of a black mouse and a pallid mouse characteristically showed the distribution of melanin pigments in the both strains; quantitative differences between these strains of mice were obvious. In black mice, the melanin granules were abundantly detected in the matrix, the medulla and the cortex of their hair follicle and in the pigment layers of the eye. These tissues were grossly black and pigments were diffusely distributed in each tissue. In pallid mice, the distribution of melanin granules was the same as in black mice. However, melanin granules were small in number; their color was light brown. Occasionally, large homogeneous melanin granules were recognized in pallid anagen hair follicles. In both strains, the epidermis was free from melanin.

Dopa reaction: The same results were obtained at both pH 6.8 and 7.4. The Dopa-reacted sections showed diffuse, heavy depositions of black to brown substance in the follicular matrix (Fig 1-*B*) and the pigment layers of eyes (Fig 1-*D*) of both black and pallid mice. Since the unstained follicular matrix (Fig 1-*A*) and ocular pigment layers (Fig 1-*C*) of pallid mice were light-colored, the Dopa-reacted tissues of pallid mice made a remarkable contrast. All control sections incubated in Dopa-free solutions showed no reaction.

Acid phosphatase reaction: The distribution of the reaction was similar in both strains. In hair follicles, heavy red precipitate was observed in the cortex at the keratogenous zone, reaction was moderate in the matrix region, the hair (dermal) papilla and the connective tissue sheath, weak in the outer and inner root sheaths; and absent in completely keratinized hair (Fig 2-*A*, 2-*B*). In the eye it was strongly positive in the pigment layers (Fig 2-*E*, 2-*F*). The reaction in these tissues was specifically inhibited with sodium fluoride (Fig 2-*C*, 2-*G*, 2-*H*).

The presence of black melanin in black tissues made it difficult to judge accurately the intensity of the reaction represented by the reddish-brown color of the precipitate. However, an impression was gained that the reaction was equal, or slightly stronger in the matrix and the cortex of pallid hair follicles (Fig 2-*B*) and the ocular pigment layers of pallid eyes (Fig 2-*F*) than in black counterparts (Fig 2-*A*, 2-*E*).

Substrate-free sections showed only pale yellow background staining.

Electron Microscopic Studies

The black hair follicle: The follicular melanocytes in black mice produced mature melanosomes which were ellipsoidal, fully melanized and about 1.0 μm long in long axis. In the cytoplasm well-developed Golgi complexes with many associated vesicles and melanosomes of all stages were observed (Fig 3-*A*). Early stage melanosomes had typical inner lamellar struc-

‡ In order to obtain an accurate tyrosinase or acid phosphatase activity in hair follicles, the hair of mice older than 10-days could not be used, because hairs of these animals were not in the same stage of growth and, in pallid mice, the hair color of old mice had already changed from pallid to white in its proximal portion.

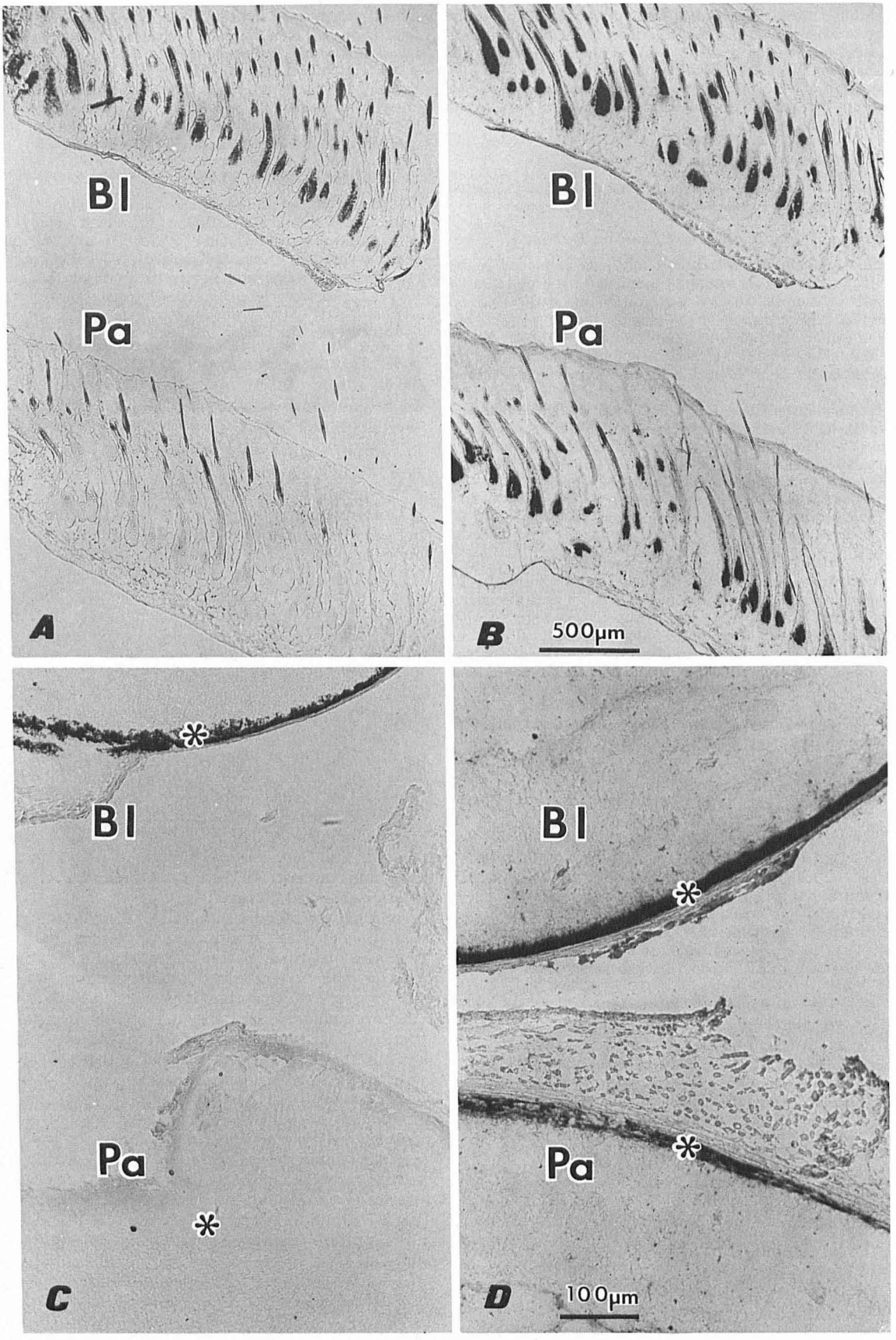

FIG 1. *A*, Untreated and unstained frozen skin sections: Notice that the hair follicles of the black mouse (*Bl*) are much darker than the pallid counterparts (*Pa*). *B*, Light microscopical *Dopa reaction* of frozen skin sections. Note a remarkable increase of density due to deposition of reaction product in the hair follicles of a pallid mouse (*Pa*). *C*, Untreated and unstained frozen eye sections. Note that pallid eye pigment layers (*Pa*, *asterisk*) are not pigmented, while black eye pigment layers (*Bl*, *asterisk*) are heavily pigmented. *D*, Light microscopical dopa reaction of frozen eye sections. Note a remarkable increase of density due to depositions of reaction product in the pigment layers (*asterisk*) of pallid eye (*Pa*). 7-day-old mice. (*A*, *B*, × 34: *C*, *D*, × 136).

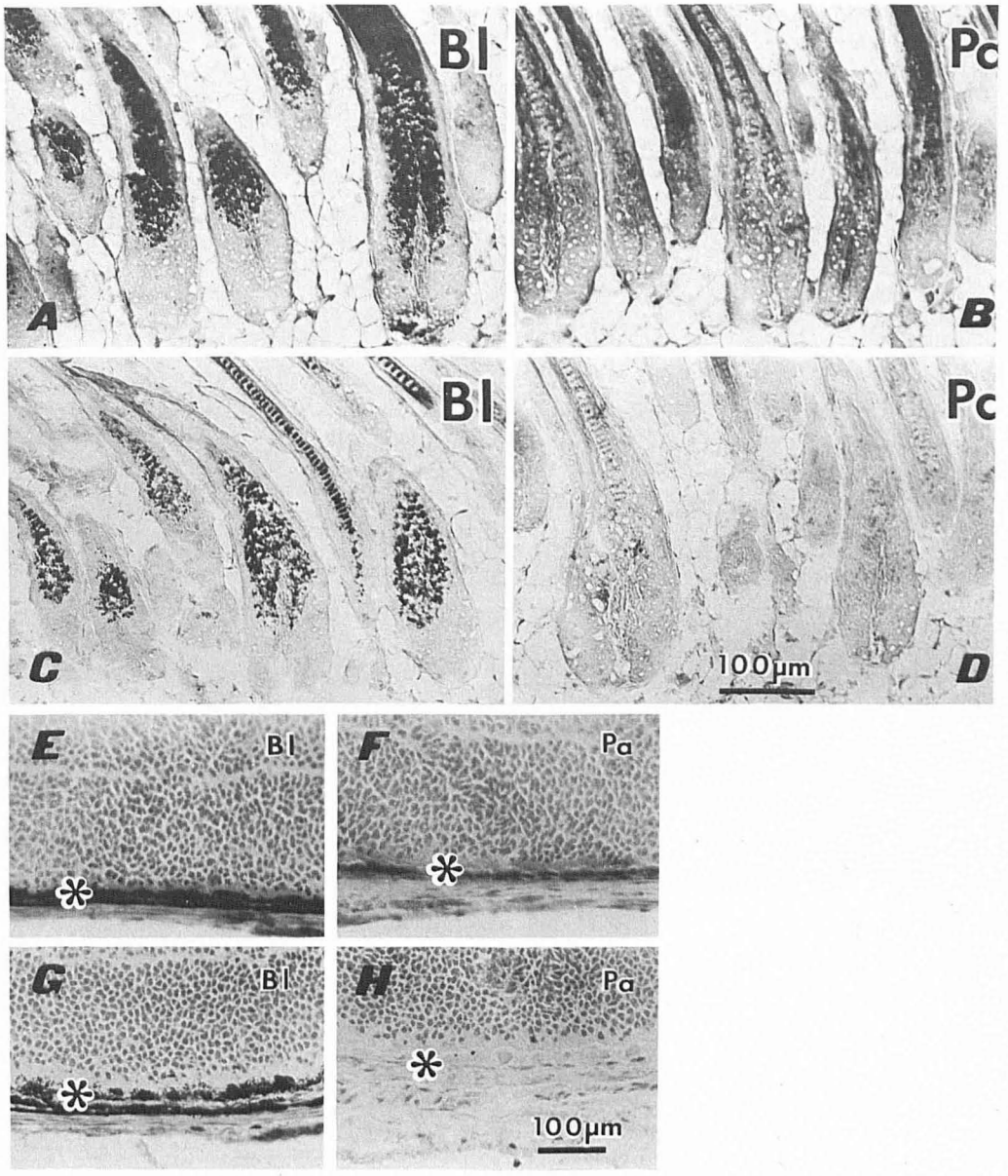

FIG 2. Light microscopical *acid phosphatase reaction* of frozen sections. *A*, Hair follicles of a black mouse. *B*, Hair follicles of a pallid mouse. *E*, Eye of a black mouse. *F*, Eye of a pallid mouse. Note the dark dense depositions of reaction products in the matrix, cortex and medulla of hair follicles and in the pigment layers (*asterisk*) of eyes. The reaction in pallid tissues (*Pa*) seems to be stronger than that in black counterparts (*Bl*). *C*, *D*, *G*, *H*: The reaction in both black (*Bl*) and pallid tissues (*Pa*) is specifically inhibited with sodium fluoride. 7-day-old mice. (*A*, *B*, *C*, *D*, × 128; *E*, *F*, *G*, *H*, × 90).

tures. Melanin deposition usually occurred evenly on those structures.

Fully-melanized melanosomes were transferred into the keratinocytes in the matrix region. The majority of the transferred melanosomes existed singly (Fig 3-*B*). They were well-preserved morphologically even at the level of the keratogenous zone (Fig 3-*C*).

The pallid hair follicle: The pallid hair follicles contained as many melanocytes as black ones. The pallid follicular melanocytes also possessed well-developed Golgi complexes with associated vesicles. They produced many melanosomes which were spherical and smaller than 0.4 μm in diameter. Although some of them were fully melanized, many of them were abnormal with irregular, uneven and varied melanin deposition. Often no melanin deposition was seen in the center of melanosomes.

There appeared to be 2 types of early stage melanosomes. One had small vesicles with or without small amounts of irregular melanin deposition, while other structure had poorly arranged and poorly developed inner lamellar structures on which uneven melamin deposition was seen. Some melanosomes having the former structure seemed to become more melanized without forming inner lamellae (Fig 3-*D*). The aggregates of 2 or 3 melanosomes were rarely recognized in pallid melanocytes.

The keratinocytes in the pallid hair matrix possessed membrane-limited aggregates (melanosome complexes) containing variously melanized organelles (Fig 3-*E*). In the keratogenous zone of the pallid hair, these melanosomal components became well-degraded (Figs 3-*F*, 3-*G*, 3-*H*). Occasionally, lamellar structures of immature melanosomes were seen even in the keratinocytes (Fig 3-*F*, 3-*H*).

Macromelanosome: On a few occasions, some melanocytes with huge round or oval macromelanosomes (2.0–5.0 μm in diameter) were found in the pallid hair follicles (Fig 4). These macromelanosomes appeared to increase their size by maturation; they never fused each other during their developmental process. They consisted of diffuse dense deposition of melanin substance inside a unit membrane and had no inner lamellar

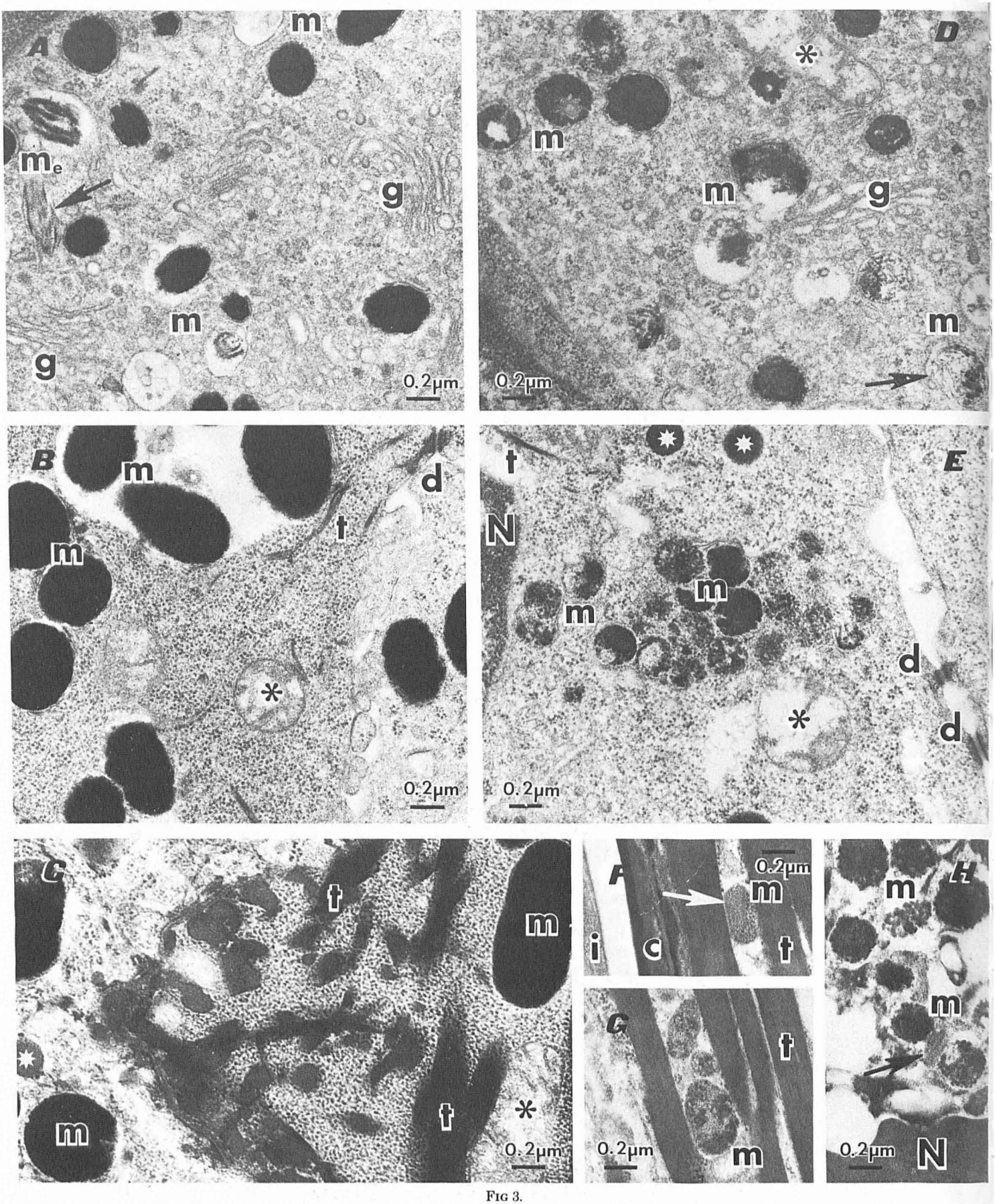
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FIG 3.

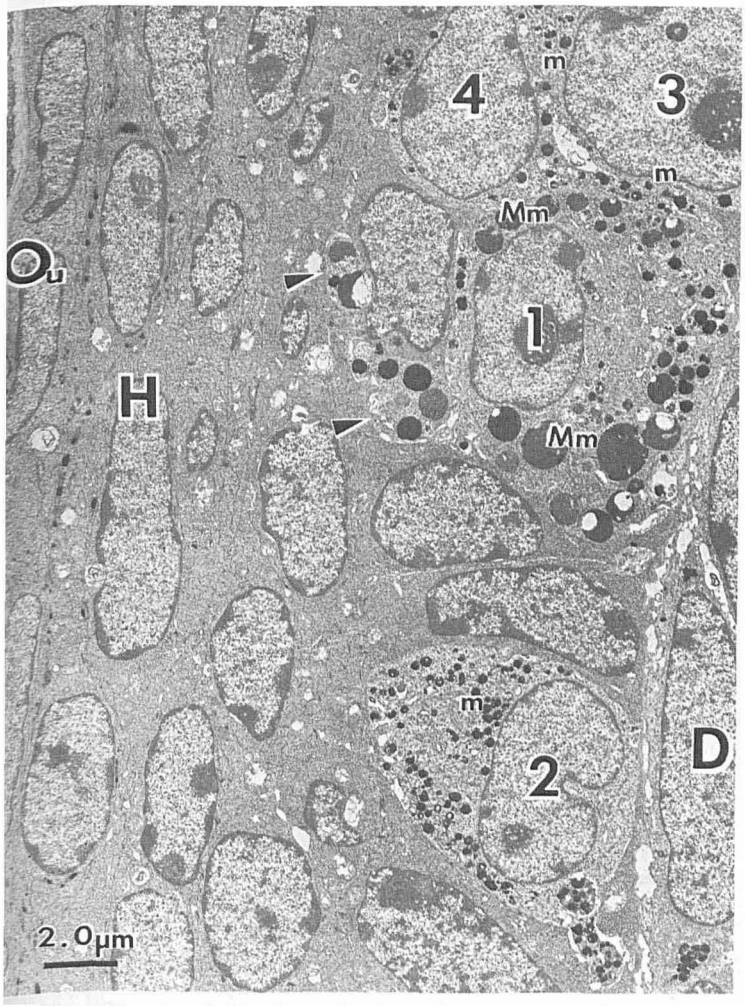

FIG 4. Electron micrograph of macromelanosomes in pallid anagen hair follicles. Lead citrate-uranyl acetate staining. Melanocyte 1 is producing macromelanosomes (*Mm*), while melanocytes 2, 3, and 4 are producing regular melanosomes (*m*) of pallid type (cf. Fig 3). *D*, hair papilla; *Ou*, outer root sheath; *H*, inner root sheath; *arrowheads*, dendrite of a melanocyte containing macromelanosomes. 120-day-old mice. (reduced from × 4,125).

structure. Morphologically, they were clearly different from the melanosome complex in the keratinocytes, which consisted of an aggregation of individual melanosomes.

The black eye: The pigment cells in the retinal pigment epithelium (RPE) and the choroid of black eyes (Fig 5-*A*) possessed fully-melanized melanosomes similar to those in black hair follicles, although the melanosomes were larger in size (about 1.5 μm in long axis) in the RPE than in the choroid (1.0 μm in long axis). The early stage melanosomes with typical inner lamellar structures were also recognized in these pigment cells of black eyes.

The pallid eye: Relatively smaller number of melanosomal structures were seen in the pigment layers of pallid eyes of 7-day-old mice (Fig 5-*B*) than in black counterparts (Fig 5-*A*). There was no difference in the distribution, number and size of the pigment cells of eyes between both strains. Most melanosomes in the RPE cells showwed ellipsoid or spindle shapes and had typical inner lamellar structures similar to those in the RPE of black eyes. These melanosomes became partially but not fully melanized (Fig 5-*C*). A small number of spherical menanosomes, which had small vesicles and some melanin depositions inside but no inner lamellae were present.

The choroid melanocyte of pallid eyes were produced spherical melanosomes (Fig 5-*D*, 5-*E*), which were morphologically very similar to those in the melanocytes of pallid hair follicles.

No macromelanosomes were found in the pigment cells of 7-day-old pallid eyes.

Dopa reaction: Electron microscopical Dopa reaction at both pH 6.8 and 7.4 showed the presence of a dopa-oxidative enzyme in the cytoplasmic vesicles and saccules near Golgi complexes and near the melanosomes in the pigment cells of hair follicles and ocular pigment layers of the black and the pallid mice. The dopa reaction of the pigment layers of pallid pigmented tissues was stronger than that of the black counterparts. The dopa reaction in the RPEs of both strains is shown in Fig 6. The controls had no positive reaction.

Acid phosphatase reaction: Both techniques showed the same result. Electron microscopic acid phosphatase reaction was positive with dense lead depositions on those melanosomal aggregates in the pallid hair keratinocytes (Fig 7-*A*, 7-*B*, 7-*C*), suggesting that lysosomal enzymes digest melanosome complexes. The vesicles and saccules near some Golgi complexes in these keratinocytes also displayed acid phosphatase reactions; however, no positive reactions were found in hair melanocytes.

In the pigment cells of pallid eyes the structures containing melanosomal components and some immature melanosomes showed acid phosphatase reactions (Fig 8-*A*, 8-*C*), suggesting that immature melanosomes were autodigested by lysosomal enzyemes in these pigment cells of eyes. Also the aggregations of melanosomes in these cells displayed acid phosphatase reactions (Fig 8-*B*, 8-*D*).

In the keratinocytes of black hair follicles and the pigment cells of balck eyes acid phosphatase reactions ere also seen in vesicles and saccules of Golgi region. Howelver, black melanosomes were not digested as readily as pallid melanosomes because of their size (Fig 3-*C*, and 5-*A*).

Macromelanosomes showed no acid phosphatase reaction. The control tissues, either inhibited with sodium fluoride or substrate-omitted, showed no dense homogeneous lead deposition, except some sparse and finely dotted lead contaminations on melanosomes.

Biochemical Studies

Assays for tyrosinase and acid phosphatase activities: It is shown in the Table that both tyrosinase and acid phosphatase activities were significantly higher in pallid hair follicles than in black hair follicles. However, the tyrosinase activity was significantly lower in pallid eye homogenates than in black ones, although pallid eye homogenates showed a higher acid phosphatase activity when compared with black ones (Table). In the tyrosinase assay 1-phenyl-2-thiourea produced almost 100% inhibition of the measured tyrosinase activity. This suggested that the activity measured in this assay was the result of tyrosinase, and not of tyrosine hydroxylase [9].

FIG 3. Electron micrographs of anagen hair follicles. Lead citrate-uranyl acetate staining. *A*, Melanocyte of black hair follicle. Note the typical lamellae in black early melanosomes (*me*). *B*, Keratinocyte near matrix of black hair follicle contains transferred, fully-melanized single melanosomes (*m*). *C*, Cortex and medulla in keratogenous zone of black hair follicle contain well-preserved melanosomes (*m*). *D*, Melanocyte of pallid hair follicle. Note the poorly-developed lamellae and uneven melanin deposition in melanosomes (*m*). *E*, Keratinocyte near matrix of pallid hair follicle. Note the melanosome complex (*m*) containing variously melanized melanosomes. *F*, *G*: Cortex and *H*: Medulla in keratogenous zone of pallid hair follicle. Note the degraded melanosome complexes (*m*). *g*, Golgi complex; *N*, nucleus; *d*, desmosome; *t*, tonofilament; *c*, cuticle of hair; *i*, cuticle of inner root sheath; *arrow*, inner lamellar structure of melanosome; *asterisk*, mitochondrion; *star*, medullary granule. 120-day-old mice. (× 33,000).

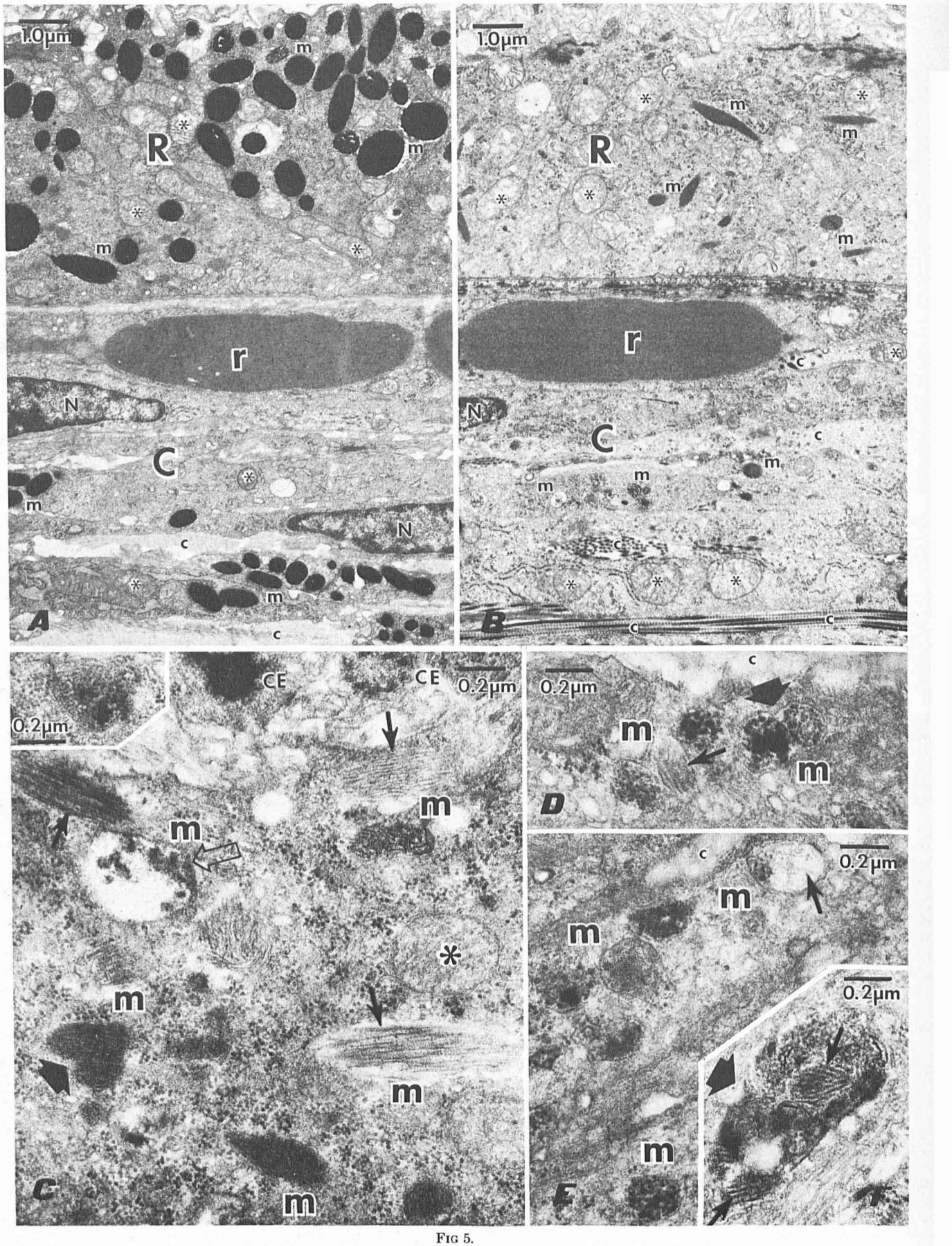
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FIG 5.

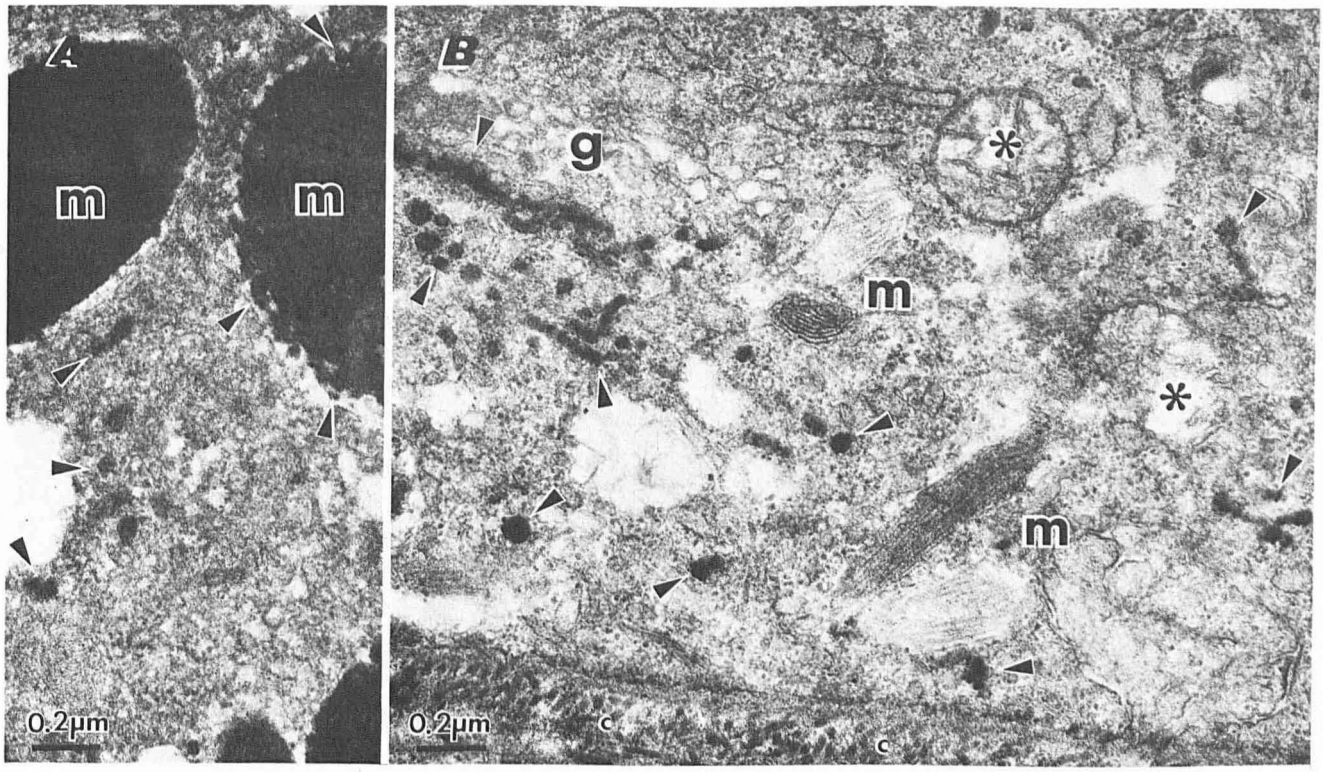

FIG 6. Electron micrographs of *dopa reaction* in the retinal pigment epithelium. Lead citrate-uranyl acetate staining. *A*, black eye. The dopa-positive vesicles (*arrowhead*) are seen in the cytoplasm and around eumelanosomes (*m*). *B*, pallid eye. Note the presence of many dopa reactive vesicles and saccules (*arrowhead*) in the cytoplasm. *g*, Golgi complex; *m*, melanosome; *asterisk*, mitochondrion; *c*, collagen fiber. (× 45,320).

DISCUSSION

Although some morphological investigations [11–13] of the melanocytes of pallid mice had been done, these studies were not detailed enough to elucidate the mechanism of coat and eye color dilution. The first possibility suggested in this study is that one type of pallid melanosome in which inner lamellar structures are poorly developed is pheomelanosome [14–16]. The second possibility raised in this study is the incompleteness of melanization in the pigment tissues of pallid hair and eye; this is probably due to immaturity or abnormality of lamellar substrate structure on which tyrosinase synthesizes melanin or to lack of essential substances for melanization. The fact that the transport of manganese, L-Dopa and L-tryptophan is slow through the tissues of pallid mice [17] might support the latter possibility. However, manganese supplementation to embryos through their mother did not change the color of coat and eye [18]. Immature melanosomes seems to be readily digested in very active lysosomes in the keratinocytes of pallid hair cortex and medulla and in the pigment cells of pallid eyes as demonstrated in this study.

Since the tyrosinase activity of pallid melanocytes in hair follicles is histochemically and biochemically greater than that of melanocytes in the black counterparts, the incomplete melanization of pallid melanosomes is due neither to low tyrosinase activity nor to tyrosinase-inhibitors. The light coat color should, therefore, be explained by the increased autophagic digestion of immature melanosomes within the melanocytes. A higher activity of acid phosphatase, a lysosomal marker, in pallid hair bulb than in black counterpart is compatible with this assumption. In the eye tissues the activity of tyrosinase was lower in pallid mice than in black mice, although by electron microscopic dopa reaction the activity of tyrosinase in pallid eyes was estimated either equal or even higher than that in black eyes because of the presence of many dopa-positive vesicles and saccules in Golgi region. This discrepancy between biochemical and ultrastructural histochemical findings could be explained as follows: A strong activity of automelanophagocytosis in pallid melanocytes as reported in this study inactivates tyrosinase activity as soon as it is incorporated into the pallid menalosomes. In black eye tissues, on the other hand, tyrosinase activity persists in mature melanosomes, thus giving a higher total value to biochemical assays using homogenate, which includes these mature melanosomes.

The digestion of melanosomes in keratinocytes is a common feature in normal diluted-colored skins [19–25], pheomelanin producing tissues [14] and abnormal hypopigmented tissues [26]. Acid phosphatase activity is present in the aggregates of melanosomes in these conditions [20,22]. It was suggested that the digestion of melanosomes results in pigment dilution of skin tissues [19].

In the pigment cells of eyes, especially the RPE cells, the

FIG 5. Electron micrographs of eyes. Lead citrate-uranyl acetate staining. *A*, Eye of a black mouse. *B*, Eye of a pallid mouse. In the retinal pigment epithelium (*R*) and the choroid (*C*), pigment cells of a black eye possess fully melanized mature eumelanosomes (*m*), whereas, those of a pallid eye possess immature melanosomes (*m*). *C*, The retinal pigment epithelium of pallid eye. Note that melanosomes (*m*), with well-arranged typical inner lamellar structures (*thin arrow*) and some melanin depositions, are never fully melanized. Some melanosomes (*empty arrow*) are melanized with small vesicles and no inner lamellar structures. *Insert*: Aggregation of melanosomes within a unit membrane. Acid phosphatase is positive in these aggregations (Fig 8-*B*). *D*, *E*, *F*: The choroid of a pallid eye. Note the similarity of melanosomes (*m*) to those in pallid hair melanocytes (Fig 3-*D*). However, these melanosomes in pallid ocular pigment cells sometimes become aggregated (*thick arrow*). *r*, red blood cell; *c*, collagen fiber; *N*, nucleus; *asterisk*, mitochondrion; *CE*, centriole; *thin arrow*, inner lamellar structure. *A*, *B*, × 9, 580; *C*, *D*, *E*, *F*, × 45,320).

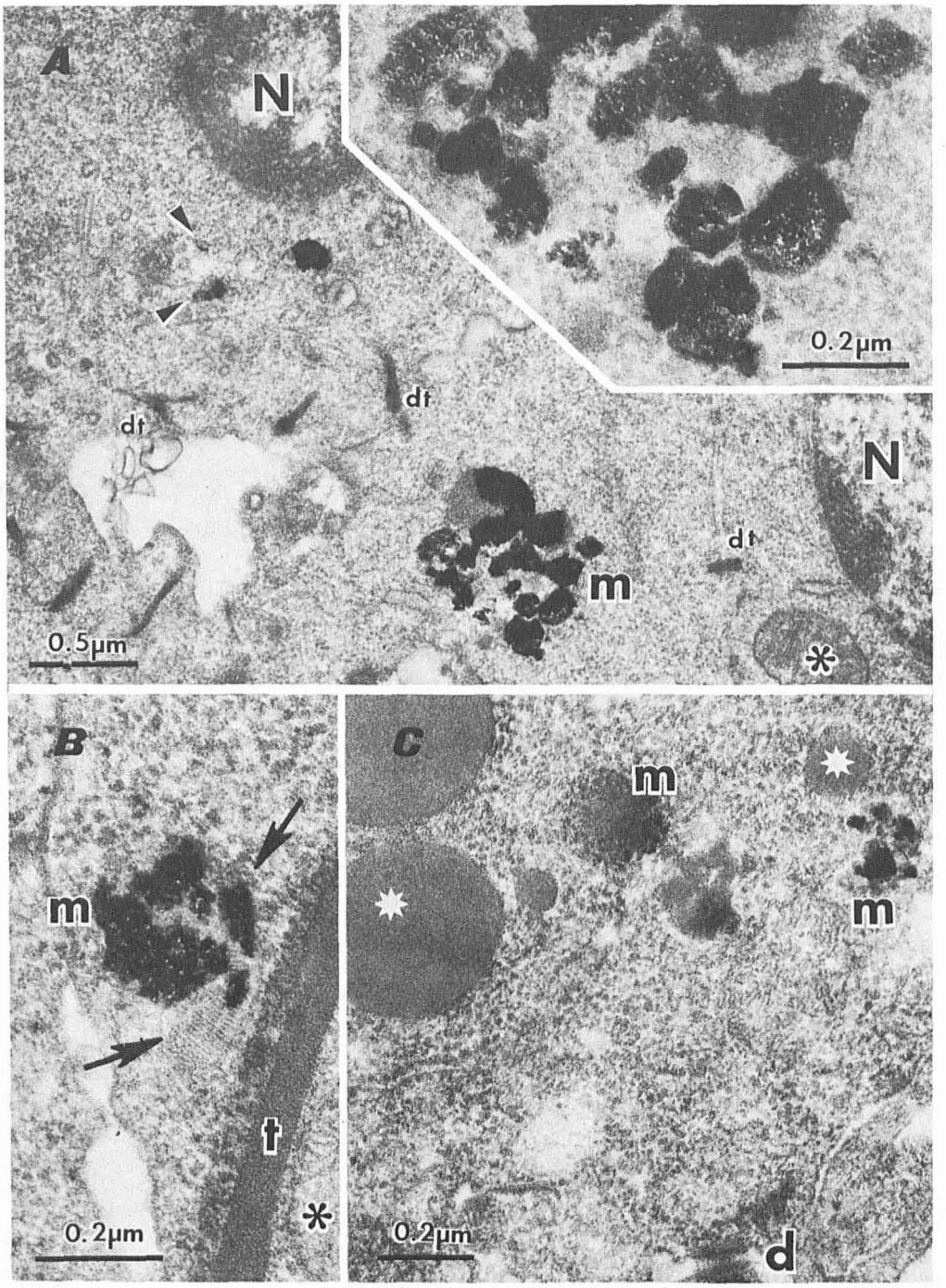

FIG 7. Electron micrographs of *acid phosphatase reaction* in keratinocytes of pallid anagen hair follicles and eye pigment cells. Lead citrate-uranyl acetate staining. *A*, keratinocytes near hair matrix. Note the dense lead depositions in vesicles (*arrowhead*) and in a melanosome complex (*m*). This melanosome complex (*m*) is magnified in the insert. Note that the dense lead depositions are present in each melanosomal component. *B*, Cortex cell. Note the dense lead depositions in a melanosome complex (*m*) with inner lamellar structures (*arrow*) of melanosomes. *C*, medulla cell. Note the dense lead depositions in single melanosomes (*m*). *dt*, desmosome with tonofilament; *d*, desmosome; *t*, tonofilaments; *N*, nucleus; *asterisk*, mitochondrion; *star*, medullary granule. 120-day-old mice. (*A*, × 30,900; *insert* of *A*, *B*, × 90,640; *C*, × 68,000).

melanosomes are known to be digested by lysosomal enzymes in such tissue as the tapetum lucidum of bovine embryo eyes [27]. A premature degradation of the melanosomes by a similar mechanism are also known in monkey fetus eyes [28]. The giant melanin granules in the eyes of beige mouse were reported to be secondary lysosomes [33,34], although their melanosomal components [29–32] were morphologically different from those of pallid mice. This study showed that the pallid immature melanosomes of ocular pigment cells were already digested by very active lysosomal enzymes at an early developmental stage (7-day-old), producing a diluted eye color. High activities of lysosomal enzymes were reported in the kidney tissue of pallid mice [33].

The macromelanosomes were found in pallid hair follicles in this study. These macromelanosomes are similar to those of nevus spilus [20,34], pigmented lesion of Leopard syndrome [35], multiple lentigines syndrome [36], lentigo simplex [37] and cafe-au-lait spots of neurofibromatosis [38]. These macromelanosomes, including pallid macromelanosomes, gradually increase their size and have no inner lamellar structure. Some

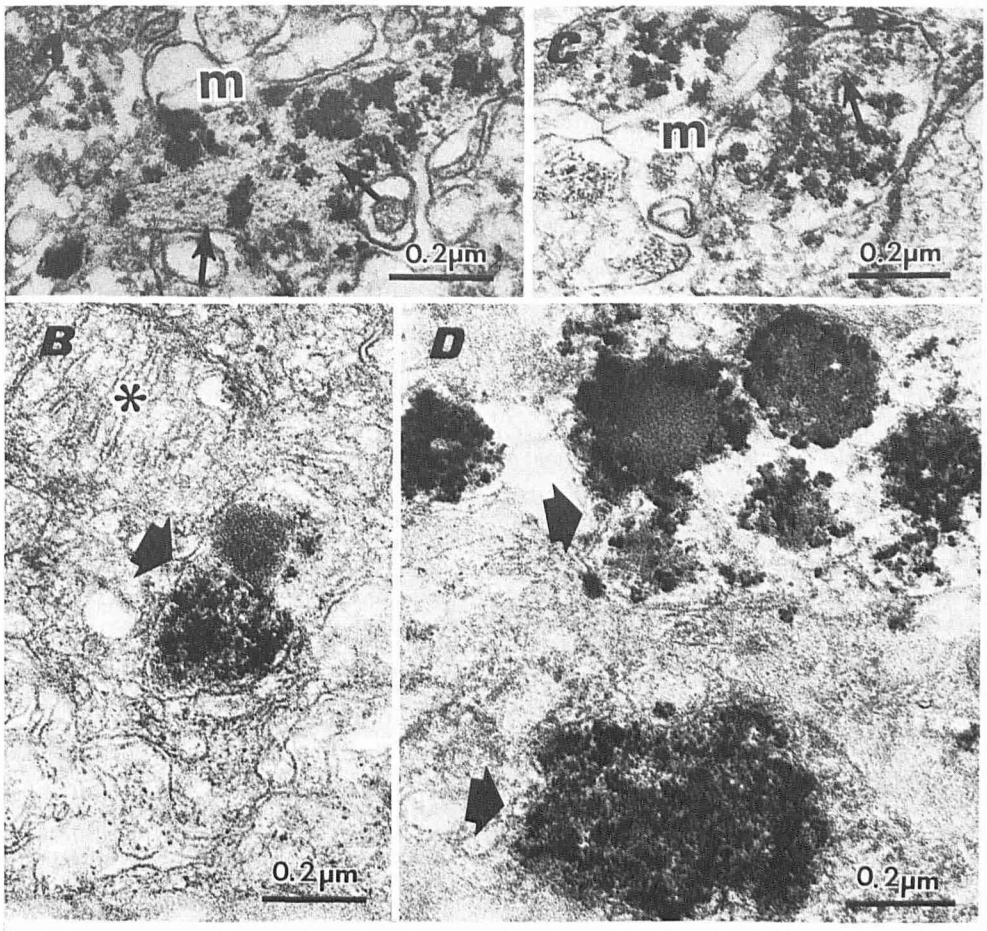

FIG 8. Electron micrographs of *acid phosphatase reaction* in pallid eye pigment cells. Lead citrate-uranyl acetate staining. *A*, *B*, retinal pigment epithelial cells. *C*, *D*, choroid cells. Note the dense lead deposit in the melanosomal structures (*m*) with inner lamellar structures (*thin arrow*) and in the aggregates of melanosomes (*thick arrow*). *Asterisk*, mitochondrion. 7-day-old mice. (× 68,000).

Tyrosinase and acid phosphatase activities of anagen hair follicles and eyes of black and pallid mice[a]

	Black hair follicle (+/Pa)	Pallid hair follicle (Pa/Pa)	Black eye (+/Pa)	Pallid eye (Pa/Pa)
Tyrosinase activity[b] (cpm/mg protein)	154,954 ± 16,997[d] (n = 6)	237,002 ± 37,422[d] (n = 6)	623,240 ± 34,075[f] (n = 4)	137,714 ± 18,479[f] (n = 4)
Acid phosphatase activity[c] (n moles/min/mg protein)	34.9 ± 5.9[e] (n = 9)	42.8 ± 6.3[e] (n = 9)	16.41 ± 0.35[g] (n^h = 5)	18.62 ± 1.02[g] (n^h = 5)

[a] Mice age, 7- and 8-day-old.
[b] L-tyrosine-3,5-^{3}H hydroxylation rate.
[c] Para-nitrophenylphosphate hydrolysis rate.
[d, e, f, g] Significant difference between 2 values in each assay by paired *t*-test: [d] $p < 0.001$; [e] $p < 0.025$; [f] $p < 0.001$; [g] $p < 0.005$.
n, number of mice.
n^h, number of eyes.

investigators [37] concluded that they were formed by complete degradation by autophagy of melanosomes within melanocytes.

We wish to thank Dr. P. Favreau and Mrs. H. M. Wang for the expert biochemical technical help.

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Announcement

Veterinary Dermatology, a symposium to be held at Stanford University School of Medicine, Department of Dermatology, August 25–27, 1982. The comparative dermatology aspect will be of interest to dermatologists as well as to veterinarians. Topics will include human and veterinary dermatology—past, present and future; fungi that attack man and animals; occupational dermatoses in veterinarians; antibiotics—use and abuse; steroids—practical considerations. In addition, the newest deficiency diseases and research in veterinary allergy will be discussed. For further information, please contact: Paul H. Jacobs, M.D., Department of Dermatology, Stanford University School of Medicine, Stanford, CA 94305; (415) 497-6101.